

Analytical verification of a multiplex PCR for identification of *Bordetella bronchiseptica* and *Pasteurella multocida* from swine

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Received 18 January 2006; received in revised form 4 May 2006; accepted 5 May 2006

Abstract

Bordetella bronchiseptica and *Pasteurella multocida* are etiologic agents of progressive atrophic rhinitis (PAR) and bronchopneumonia in swine. Only dermonecrotic toxin-producing strains of *P. multocida* play a role in atrophic rhinitis while both toxigenic and nontoxigenic strains have been associated with pneumonia. Monitoring and investigation of outbreaks involving these bacteria require sensitive and accurate identification and reliable determination of the toxigenic status of *P. multocida* isolates. In the present study, we report the development, optimization, and performance characteristics of a multiplex PCR assay for simultaneous amplification of up to three different targets, one common to all *P. multocida* strains, one found only in toxigenic *P. multocida* strains, and one common to *B. bronchiseptica* strains. Based on analysis of 94 *P. multocida* isolates (31 toxigenic) and 126 *B. bronchiseptica* isolates assay sensitivity is 100% for all amplicons. Evaluation of 22 isolates of other bacterial genera and species commonly found in the swine respiratory tract demonstrated a specificity of 100% for all gene targets. The limit of detection for simultaneous amplification of all targets is 1–10 pg of DNA per target, corresponding to a few hundred genomes or less. Amplicon mobility in agarose gels and sequence analysis indicate the amplicons are highly stable. The data presented establish this multiplex PCR as a reliable method for identification of *B. bronchiseptica* and both toxigenic and nontoxigenic *P. multocida* that may greatly simplify investigations of swine PAR and bronchopneumonia.

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Keywords: *Bordetella bronchiseptica*; *Pasteurella multocida*; PCR; Atrophic rhinitis

1. Introduction

Bordetella bronchiseptica and *Pasteurella multocida* are etiologic agents of pneumonia and progressive atrophic rhinitis (PAR) in swine (de Jong, 2006). Monitoring and investigation of outbreaks requires sensitive and accurate identification of these bacteria.

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Determination of the toxigenic status of *P. multocida* is of importance for diagnosis of PAR, since only strains that produce a dermonecrotic toxin, PMT, play a role in that disease (de Jong, 2006).

Detection of *B. bronchiseptica* and toxigenic/nontoxigenic *P. multocida* is typically based on isolation and biochemical testing of suspect colonies cultured from swabs or biopsies, an approach which is time-consuming, cumbersome, and suffers from poor sensitivity. Probe-based methods, although highly specific and sensitive (Kamps et al., 1990a; Register et al., 1995, 1998), are not readily incorporated into the workload of a diagnostic laboratory. In contrast, PCR is increasingly being implemented in diagnostic settings and often offers superior performance. Numerous investigators have described PCRs for detection of toxigenic *P. multocida* from swine (Hunt et al., 2000; Donnio et al., 1999) or for detection of all strains without regard to toxigenic status (Hunt et al., 2000; Miflin and Blackall, 2001; Liu et al., 2004). One *B. bronchiseptica*-specific PCR has been described (Hozbor et al., 1999), but it was not evaluated for veterinary applications. Additional analysis is required before this assay can be recommended for use in investigations of swine atrophic rhinitis and bronchopneumonia. A single reliable method for identification of *B. bronchiseptica* and both toxigenic and nontoxigenic *P. multocida* would greatly simplify investigations of swine PAR and bronchopneumonia. The goals of the present study were to design and optimize a multiplex PCR for this purpose and to establish its performance characteristics.

2. Materials and methods

2.1. Bacterial isolates

P. multocida strains 4533 (toxigenic) and 1059 (nontoxigenic) were used as controls (Rimler and Brogden, 1986). Ninety-four additional *P. multocida* isolates from pigs (Table 1) were obtained from the National Animal Disease Center collection. These strains originated from diverse geographic locations and over a broad span of time (approximately 8% between 1958 and 1979, 48% during the 1980s, and 44% between 1998 and 2002). Identification was based on standard biochemical testing and colony

Table 1
Bacterial isolates included in this study

Isolate	Geographic origin	n
<i>B. bronchiseptica</i>	United States, Canada	62
	Continental Europe	47
	Ireland, United Kingdom	15
	Australia	2
<i>P. multocida</i>		
Nontoxigenic	United States, Canada	53
	South America	2
	United Kingdom	1
	Norway	3
	Sweden	2
	Malaysia	2
Toxigenic	United States, Canada	18
	Norway	7
	Sweden	5
	Singapore	1
<i>Mycoplasma</i>	United States	6
<i>hyopneumoniae</i>		
<i>Mycoplasma hyorhinis</i>	United States	4
<i>Mycoplasma flocculare</i>	United States	4
<i>Haemophilus parasuis</i>	United States	3
<i>Actinobacillus suis</i>	United States	1
<i>Salmonella choleraesuis</i>	United States	1
<i>Arcanobacterium</i>	United States	1
<i>pyogenes</i>		
<i>Actinobacillus</i>	United States	1
<i>pleuropneumoniae</i>		
<i>Streptococcus suis</i>	United States	1

morphology. The capsular type of 62 of the isolates was previously determined for other studies, using either a passive hemagglutination test and type-specific sera (Rimler and Brogden, 1986) or a multiplex PCR typing assay (Townsend et al., 2001; G. Nordholm, unpublished data); 29% are type A and 71% are type D. A colony-blot assay using a PMT-specific monoclonal antibody was used to classify *P. multocida* strains as either toxigenic or nontoxigenic (Magyar and Rimler, 1991). Isolates were grown at 37 °C for 24 h on Dextrose Starch Agar.

B. bronchiseptica strain KM22 (Brockmeier et al., 2000) was used as a control. One hundred twenty-six additional *B. bronchiseptica* swine isolates, from a variety of locations worldwide, were evaluated (Table 1). All were originally identified based on culture characteristics and standard biochemical testing. Those originating within the United States were from the National Animal Disease Center

collection. The remainder were provided either by J.M. Musser (Musser et al., 1987), G. Foster (SAC Veterinary Services, Drummond Hill, Inverness, Scotland) or T. Magyar (Hungarian Academy of Sciences, Budapest, Hungary). All isolates were confirmed to be *B. bronchiseptica* based on ribotyping; they represent all *Pvu*II ribotypes so far identified from swine (Register et al., 1997; Register and Magyar, 1999). Approximately 43% were obtained in the 1980s, 49% in the 1990s, and the remainder since 2000. Cultures were grown at 37 °C for 36–48 h on Bordet-Gengou agar supplemented with 10% sheep's blood.

Other bacteria included in this study were obtained from the National Animal Disease Center collection or were kindly provided by E. Thacker or R. Griffith, Iowa State University, Ames, IA, USA.

2.2. PCR

2.2.1. Template preparation

Single colony lysates were the source of template for most PCRs. A colony ~2 mm in diameter was suspended in 10 µl of water and boiled for 5 min. Following brief centrifugation to pellet cell debris, 1 µl of the supernatant was added to the PCR mix.

Template used to establish the limit of detection based on CFU was derived from bacteria suspended in PBS to an A_{600} of 0.42. Aliquots from a series of dilutions were boiled as described above and added directly to PCRs.

Chromosomal DNA, used to establish the limit of detection or compare amplification efficiency, was purified with a commercially available kit (Gentra Systems, Minneapolis, MN, USA). DNA was quantitated with PicoGreen (Molecular Probes, Eugene, OR, USA), a highly sensitive fluorescent stain specific for double-stranded DNA.

PCR was carried out in an Applied Biosystems (Foster City, CA, USA) 9700 thermal cycler. Ten microliters of each PCR was analyzed by agarose gel electrophoresis in 3:1 NuSieve (Cambrex BioScience Rockland Inc., Rockland, ME, USA) containing 0.5 µg/ml ethidium bromide.

2.2.2. Single locus PCR

The strategy utilized for development of a multiplex PCR was that described by Henegariu et al. (1997). The protocol first requires identification of

primers for sensitive and specific single locus PCR under a standard set of conditions. The primers KMT1T7 (forward) and KMT1SP6 (reverse; Townsend et al., 1998), which amplify a 457 bp fragment from the *kmt1* gene, were evaluated for species-specific detection of *P. multocida*. Four primers were assessed in various combinations for detection of the gene encoding PMT, *tox*A. Two, referred to here as *tox*A-1 (forward) and *tox*A-2 (reverse), have been previously described and were originally designated oligonucleotide 1 and oligonucleotide 4, respectively (Nagai et al., 1994). The Primer Design module of Vector NTI Advance (Invitrogen, Carlsbad, CA, USA) was used to select an additional forward primer (*tox*A-7; 5'-ACTACAGATTCCTAACAAAGGTTCTGG-3') and reverse primer (*tox*A-6; 5'-TGCTCAAATCCTAAATCACCTTGT-3'). For identification of *B. bronchiseptica* two primer sets were evaluated. The primers Fla4 (forward) and Fla2 (reverse), which flank a 237 bp region upstream of the *B. bronchiseptica* flagellin gene *flaA*, have been described (Hozbor et al., 1999). The Vector Primer Design module was used to select an alternate set targeting the alcaligin gene, *alcA*, based on the prior demonstration that a hybridization assay utilizing an *alcA* probe is highly sensitive and specific for detection of *B. bronchiseptica* (Register et al., 1995, 1998). The forward primer *alcA*-1 (5'-CGCATTATTCTACTACATGCG-3') and reverse primer *alcA*-2 (5'-GACTATCTGCGC-CATAAATACGAA-3') amplify a 309 bp fragment from *alcA* (GenBank accession number U32117). Primer compatibility was evaluated with the MultiPLX program (Kaplinski et al., 2005) under normal stringency conditions.

A hot start PCR protocol, based on the use of AmpliTaq Gold and PCR Buffer II (Applied Biosystems, Foster City, CA, USA), was carried out under conditions recommended by the manufacturer (1.25 U AmpliTaq Gold, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 200 µM dNTPs, 0.5 µM primers, and 2.5 mM MgCl₂, in a final volume of 50 µl). In preliminary experiments with individual primer sets the following cycling conditions were found to reliably amplify all fragments: 10 min at 95 °C, 25 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s, and a final extension step of 72 °C for 7 min. All single locus PCRs performed comparably in the presence of MgCl₂ concentrations ranging from 1.5 to

3.0 mM; 2.5 mM was arbitrarily adopted as standard protocol.

A previously described 16S rRNA-specific PCR was carried out with all samples (Register and Yersin, 2005). The forward primer (5'-AGAGTTTGA-TCCTGGCTCAG-3'), designated univ16S-3, is homologous to highly conserved sequence from the 5' end of the 16S rRNA gene. The reverse primer (5'-GCGGCTGCTGGCAG-3'), designated univ16S-4, is derived from highly conserved sequence between the third and fourth variable regions of the gene. This primer set generates an amplicon of approximately 520 bp from all bacteria with which it has so far been tested, including a variety of genera and species of veterinary importance (Register and Yersin, 2005; unpublished data). Reactions were carried out in a volume of 25 μ l and contained 1 U AmpliTaq polymerase (Applied Biosystems, Foster City, CA, USA), 200 μ M dNTPs, 0.5 μ M primers, 1.5 mM MgCl₂, and 2.5 μ l of 10 \times Buffer II. An initial denaturation step of 1 min at 95 °C was followed by 35 cycles of 1 min at 95 °C, 30 s at 53 °C, and 30 s at 72 °C, with a final extension step of 5 min at 72 °C.

2.2.3. Multiplex PCR

Cycling and reactant conditions initially used for multiplex PCR were identical to those detailed above, except that 10 additional cycles were added (a total of 35 cycles) and all primers were present at 0.3 μ M. In some experiments 10 \times PCR Gold Buffer (Applied Biosystems, Foster City, CA, USA), consisting of 150 mM Tris-HCl, pH 8.0, and 500 mM KCl, was substituted for 10 \times PCR Buffer II. Further optimization of reactant conditions is described below.

2.2.4. Performance characteristics

PCR performance characteristics were established based on standardized guidelines (Hoorfar and Cook, 2003; Nolte and Caliendo, 2003). Assay sensitivity was defined as true positives/(true positives + false negatives) \times 100%. Assay specificity was defined as true negatives/(true negatives + false positives) \times 100%.

The limit of detection per mass of DNA was determined using a series of 10-fold serial dilutions of purified DNA from either *P. multocida* strain 4533 (primer sets toxA-6/toxA-7 and KMT1T7/KMT1SP6), *P. multocida* strain 3480 (primer set KMT1T7/

KMT1SP6) or *B. bronchiseptica* strain KM22 (primer set Fla4/Fla2). The data were converted to theoretical numbers of bacteria based on the currently reported genome sizes for *P. multocida* (May et al., 2001) and *B. bronchiseptica* (Parkhill et al., 2003) and standard mass conversion factors.

The limit of detection per CFU was also assessed for individual primer sets. Aliquots of serial 10-fold dilutions in PBS were prepared from bacterial suspensions and either boiled and used as templates in PCRs as described above or spread, in duplicate, on 10% sheep's blood agar plates. Following incubation of plates at 37 °C for 24 h (*P. multocida*) or 36 h (*B. bronchiseptica*) the average number of CFU was determined.

2.3. DNA sequencing

PCR products were purified with spin columns (Qiagen, Valencia, CA, USA) and sequenced directly by fluorescence-based cycle sequencing with AmpliTaq and BigDyeTM Terminators on an ABI 377 sequencer, at the National Animal Disease Center Genomics Unit. Sequences were analyzed using Vector NTI Suite software (Invitrogen, Carlsbad, CA, USA). Final sequences represent a minimum of three reactions with at least one from each strand. GenBank accession numbers for sequences reported here are DQ367747–DQ367777.

3. Results

3.1. Evaluation and performance characteristics of single locus primer sets

Development of a multiplex PCR assay for identification of *P. multocida* and *B. bronchiseptica* which also discriminates between toxigenic and nontoxigenic strains of *P. multocida* first requires that individual gene targets with high sensitivity and specificity be identified. Therefore, we initially evaluated primer sets separately under a standard set of conditions for detection of either (1) *P. multocida*, (2) toxigenic *P. multocida* or (3) *B. bronchiseptica*.

The primers KMT1T7/KMT1SP6 (Townsend et al., 1998), which target a gene of unknown function with

high sensitivity and specificity for *P. multocida*, were tested here with the 94 *P. multocida* swine isolates included in Table 1. A product of the expected size was amplified from all isolates, confirming the assay sensitivity as 100%. Primer specificity was assessed using 9 additional species of bacteria commonly found in swine, representing 8 genera, as well as 15 of the *B. bronchiseptica* isolates listed in Table 1. There was no detectable PCR product from any of these reactions, although a fragment was generated from all isolates using the univ16S-3/univ16S-4 primer pair as a positive control, thereby establishing the KMT1T7/KMT1SP6 primer specificity as 100%.

As already noted, several PCR assays for detection of toxigenic strains of *P. multocida* from swine have been described, targeting various regions of the 3858 bp *toxA* gene (Hunt et al., 2000; Donnio et al., 1999). Since those with the highest concordance among all data amplify the central portion of the gene, two forward and two reverse primers derived from sequence within this region were selected for preliminary testing with a subset of the toxigenic and nontoxigenic *P. multocida* strains listed in Table 1. Based on data obtained using the primers in various combinations the pair *toxA*-7 (forward primer)/*toxA*-6 (reverse primer), which amplifies a 498 bp fragment, was selected as optimal and used for further testing. An amplicon of the appropriate size was generated from lysates of all toxin-producing isolates included in this study. No amplicon was detected from either the 63 nontoxigenic *P. multocida* strains or from the other genera and species of bacteria evaluated (Table 1). A single fragment was generated from all lysates in PCRs with the positive control primer set univ16S-3/univ16S-4. These data establish both specificity and sensitivity of the *toxA*-7/*toxA*-6 PCR as 100%.

Two different PCRs were initially compared for detection of *B. bronchiseptica*: (1) the species-specific Fla4/Fla2 PCR (Hozbor et al., 1999), which amplifies a region upstream of the flagellin gene *flaA* and (2) a PCR specific for *alcA*, an alcaligin synthesis gene found in multiple *Bordetella* species (Giardina et al., 1995; Kang et al., 1996; Parkhill et al., 2003) but previously shown to provide a highly sensitive target for probe-based identification of *B. bronchiseptica* from pigs (Register et al., 1995, 1998). Preliminary comparative analysis using 15 of the *B. bronchiseptica* isolates included in Table 1 resulted in a single

amplicon of the predicted size for each PCR from all isolates. The amplification efficiency of each assay appeared similar, since there was no noticeable difference in the amplicon yield based on band intensity in agarose gels when equivalent amounts of purified genomic DNA were included as template. The Fla4/Fla2 PCR was selected for additional analysis on the basis of its species specificity. A product of the expected size was amplified from the remaining 111 *B. bronchiseptica* isolates included in this study. Primer specificity was evaluated with 15 of the *P. multocida* isolates as well as the remaining bacteria listed in Table 1. No product was detected from any reaction. However, an amplicon was generated from all lysates using the univ16S-3/univ16S-4 primers. These data demonstrate both the sensitivity and specificity of the Fla4/Fla2 PCR to be 100%.

The results of PCRs with individual primer sets using either 25, 30, or 35 cycles were compared. Thirty-five cycles appeared optimal for all three primer sets, since the intensity of the amplicons was noticeably increased without appearance of secondary products. This cycle number was adopted as standard protocol.

The limit of detection for each primer set was initially determined individually using a series of 10-fold dilutions of purified DNA from *P. multocida* strain 4533 (for KMT1T7/KMT1SP6 and *toxA*-7/*toxA*-6) or *B. bronchiseptica* strain KM22 (for Fla4/Fla2). Between 1 and 10 pg of DNA was required for visualization of the KMT1T7/KMT1SP6 amplicon or the Fla4/Fla2 amplicon, while the limit of detection for the *toxA*-7/*toxA*-6 amplicon was between 1 and 0.1 pg (Fig. 1). Using currently available data from *P. multocida* (May et al., 2001) and *B. bronchiseptica* (Parkhill et al., 2003) genome sequencing projects, the limit of detection was calculated as being in the range of 390–3900 bacteria for KMT1T7/KMT1SP6, 39–390 bacteria for *toxA*-7/*toxA*-6, and 165–1650 bacteria for Fla4/Fla2.

Since bacterial lysates contain components other than DNA which could potentially alter amplification efficiency, the limit of detection for each primer set based on numbers of CFU found in serially diluted suspensions was also determined. Limits of detection were 10–100 CFU for the KMT1T7/KMT1SP6 amplicon, 2–20 CFU for the *toxA*-7/*toxA*-6 amplicon, and 70–700 CFU for the Fla4/Fla2 amplicon. Substitution

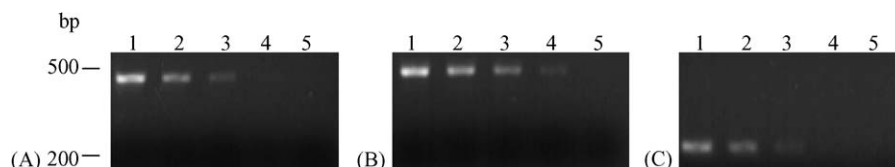


Fig. 1. Limit of detection based on mass of purified DNA for PCR using primers KMT1T7/KMT1SP6 (A), toxA-7/toxA-6 (B) or Fla4/Fla2 (C) and decreasing amounts of template. Lane 1: 1000 pg; lane 2: 100 pg; lane 3: 10 pg; lane 4: 1 pg; lane 5: 0.1 pg.

of a nontoxicogenic *P. multocida* strain in PCRs with the KMT1T7/KMT1SP6 primer set did not significantly affect the limit of detection. These results are consistent with those obtained using purified DNA and suggest the presence of additional bacterial components does not adversely affect the limit of detection.

3.2. Optimization of multiplex PCR

Prior to testing the KMT1T7/KMT1SP6, toxA-7/toxA-6, and Fla4/Fla2 primers together in a single PCR, they were evaluated for compatibility using the MultiPLX program (Kaplinski et al., 2005). This analysis generates compatibility scores based on possible interactions among all pairwise combinations of primers and products and provides a detailed description of any predicted problem pairs which break predetermined cut-off values. No problematic interactions were identified under the biochemical conditions used and over a range of MgCl_2 concentrations (1.0–4.0 mM).

According to the recommendations of Henegariu et al. (1997), the multiplex PCR was initially carried out with an equimolar primer mix (0.3 μM each). When toxigenic *P. multocida* and *B. bronchiseptica* boiled lysates were included as templates the three expected bands were usually detectable, but fluorescence of the Fla4/Fla2 amplicon was consistently weaker than either of the *P. multocida* targets. Amplification of genes from *Bordetella* species is often enhanced in the presence of dimethyl sulfoxide (DMSO) due to the high G + C content of the genomes of this genera (Parkhill et al., 2003). Although inclusion of 10% DMSO in the multiplex PCR did improve the yield of the Fla4/Fla2 product, there was a concomitant decrease in the fluorescence intensity of the toxA-specific amplicon. Following additional manipulation of DMSO and primer concentrations, optimal results were obtained using 5% DMSO,

0.3 μM KMT1T7/KMT1SP6, 0.3 μM toxA-7/toxA-6, and 0.5 μM Fla4/Fla2. These concentrations were used for all subsequent evaluation.

3.3. Multiplex PCR limit of detection

The limit of detection for the multiplex PCR was determined with template for all amplicons included in the reaction, since amplification of individual fragments is likely to be least efficient under these circumstances. When 10 pg each of *P. multocida* strain 4503 (toxigenic) and *B. bronchiseptica* were present all amplicons were readily apparent. Although difficult to capture in photographs, products generated from as little as 1 pg could be faintly visualized when the gel was viewed directly on a UV lightbox. Accordingly, the multiplex PCR can be expected to demonstrate the presence of a few to several hundred *P. multocida* and/or *B. bronchiseptica* genomes.

Based on the recommendation of the manufacturer, 10 \times Gold Buffer was substituted for 10 \times PCR Buffer II in an effort to further enhance the limit of detection. Amplification using Gold Buffer appeared to be less robust, since the limit of detection was approximately 25 pg per amplicon under these conditions.

3.4. Multiplex PCR reproducibility

Reproducibility of the multiplex PCR was evaluated with cell lysates from all toxigenic *P. multocida* and roughly half the nontoxicogenic *P. multocida* and *B. bronchiseptica* isolates in this study, two to five times over a period of 18 months. There was 100% concordance among reactions prepared from replicate lysates of the same isolate. Complete agreement of replicate PCRs was also observed when lysates stored at 4 $^{\circ}\text{C}$ for up to 1 week were retested. Additionally, there was 100% concordance of negative results with

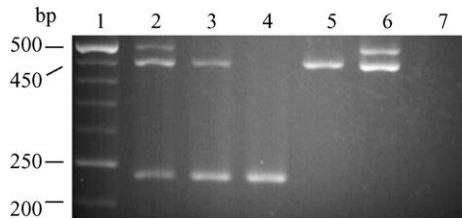


Fig. 2. Agarose gel electrophoresis demonstrating possible outcomes of the KMT1T7/KMT1SP6, *toxA-7/toxA-6*, *Fla4/Fla2* multiplex PCR. Reactions contained lysates made with toxigenic *P. multocida* and *B. bronchiseptica* (lane 2), nontoxigenic *P. multocida* and *B. bronchiseptica* (lane 3), *B. bronchiseptica* (lane 4), nontoxigenic *P. multocida* (lane 5) or toxigenic *P. multocida* (lane 6). Lanes 1 and 7 contain molecular mass marker DNA fragments or a negative control PCR with water in place of template, respectively.

the other genera and species of bacteria included in this study when PCRs were repeated from either freshly made or stored lysates.

PCRs containing lysates with various mixtures of *P. multocida* and/or *B. bronchiseptica* were also carried out many times over a period of 24 months. Results were as expected, based on the isolate(s) included in the reactions. Representative examples illustrating

possible outcomes of the PCR are shown in Fig. 2. The PCR will not discriminate between samples containing toxigenic *P. multocida* and those containing both toxigenic and nontoxigenic *P. multocida* unless testing is carried out separately on individual colonies.

3.5. Sequence analysis of *toxA-7/toxA-6* amplicons

No alterations were noted in the mobilities of either the KMT1T7/KMT1SP6, *toxA-7/toxA-6*, or *Fla4/Fla2* amplicons generated from different isolates, suggesting the targeted sequences are stable. Nonetheless, we determined the DNA sequence of the 31 *toxA-7/toxA-6* amplicons generated in this study. All sequences are 100% identical to one another as well as to the corresponding region of four out of six *toxA* sequences currently available in GenBank (Fig. 3, variant A). Variant B contains a single conservative bp substitution (C for T) that maintains the serine at amino acid position 707 of PMT. Variant C has two consecutive bp substitutions predicted to result in replacement of an alanine at amino acid position 775 with an arginine. It is not clear precisely how or whether this substitution,

variant A	2077	TTTTTACTTC	GTACAGCCAT	GAATGAAATG	GCTGGAAAAA	CCAGTGAAAG	CACGGCTGAT
variant B		-----	-----	-----	-----	-----C-----	-----
variant C		-----	-----	-----	-----	-----	-----
variant A	2137	TTAATACGCT	TTGCCTTGCA	AGATACAGTA	ATTTCAGCGC	CTTTTCGCGG	ATATGCTGGT
variant B		-----	-----	-----	-----	-----	-----
variant C		-----	-----	-----	-----	-----	-----
variant A	2197	GCGATTCCAG	AGGCAATAGA	CTTTCCTGTA	AAATATGTAA	TAGAAGACAT	ATCTGTATTT
variant B		-----	-----	-----	-----	-----	-----
variant C		-----	-----	-----	-----	-----	-----
variant A	2257	GATAAAATAC	AGACAAATTA	CTGGGAACCT	CCTGCTTATG	AAAGCTGGAA	CGAAGGAAGT
variant B		-----	-----	-----	-----	-----	-----
variant C		-----	-----	-----	-----	-----	-----
variant A	2317	AATAGCGCAT	TACTGCCTGG	TTTGTTACGT	GAATCGCAAA	GCAAGGGGAT	GTTAAGTAAG
variant B		-----	-----	-----	-----	-----	-----
variant C		-----CG--	-----	-----	-----	-----	-----
variant A	2377	TGTCGTATCA	TAGAAAATAG	CCTTTATATT	GGACATAGCT	ATGAAGAAAT	GTTTTACAGC
variant B		-----	-----	-----	-----	-----	-----
variant C		-----	-----	-----	-----	-----	-----
variant A	2437	ATTCTCCAT	ATTCAAACCA	GGTTGGAGGG	CCTTATGAAT	TATATCCTTT	CAC
variant B		-----	-----	-----	-----	-----	---
variant C		-----	-----	-----	-----	-----	---

Fig. 3. DNA sequence variants of the *toxA-7/toxA-6* amplicon. Dashes indicate conserved bases. Substitutions are indicated by the appropriate letter, as compared to the sequence defined as variant A. Base positions are numbered relative to the first base of the *toxA* start codon.

which replaces a hydrophobic, nonpolar amino acid with a hydrophilic, basic amino acid, might affect the structure or function of PMT. It was noted that the base substitutions reported in variant C constitute an inversion of the sequence found in all other isolates to date. Based on the limited sequence heterogeneity observed, we conclude the *tox*A-7/*tox*A-6 amplicon is a highly stable target.

4. Discussion

The sensitivity of the KMT1T7/KMT1SP6 and Fla4/Fla2 primer sets was previously determined to be 100% (Townsend et al., 1998; Hozbor et al., 1999), although 30 or fewer *P. multocida* and *B. bronchiseptica* isolates were evaluated and it is unclear whether any were obtained from swine. Our evaluation of a large and diverse group of both *P. multocida* and *B. bronchiseptica* swine isolates demonstrates that the gene targets for all three primer sets selected for the multiplex PCR are present in a wide variety of strains. Assay sensitivity is also critically dependent upon sequence stability of the primer-binding regions. Little sequence variation was anticipated in either the *B. bronchiseptica* target, which comprises a portion of the promoter region for the flagellin gene, or the *P. multocida*-specific target *kmt1*, inferred to be a hydrolase by electronic annotation (<http://us.expasy.org/cgi-bin/niceprot.pl?Q9CL81>). This expectation is supported both by comparison of amplicon mobilities in agarose gels and sequence analysis of amplicons from a limited number of strains (unpublished data). Although no alterations in mobility were likewise noted for the *tox*A-7/*tox*A-6 amplicons, they were more thoroughly scrutinized since *tox*A codes for the major protective immunogen in PAR. Bacterial genes coding for protective antigens are particularly prone to the accumulation of sequence substitutions, deletions, and/or insertions in response to immunological or other host-specific selective pressures. Furthermore, of the six *tox*A sequences currently available for comparative analysis three unique variants can be identified within the region corresponding to the *tox*A-7/*tox*A-6 amplicon. This suggests considerable heterogeneity may exist within the amplicon sequence from different strains, even though the major antigenic epitopes of PMT appear to reside within other regions

of the gene (Petersen et al., 1991; Liao et al., 2006). Nonetheless, our analysis revealed all *tox*A-7/*tox*A-6 sequences from the isolates included in this study are identical, confirming the suitability of this portion of *tox*A as a reliable and highly sensitive target for diagnostic PCR.

The specificity of KMT1T7/KMT1SP6 and Fla 4/Fla 2 was also addressed in previous studies. Regarding KMT1T7/KMT1SP6, only 1 false positive result occurred (with *Pasteurella canis* biotype 2) in an analysis that included several different species of *Pasteurella* as well as bacteria representing eight other genera (Townsend et al., 1998). Considering that data as well as the results reported here, an overall specificity of 98.2% can be expected with the KMT1T7/KMT1SP6 primer set (1 false positive out of 55 isolates tested, representing 12 genera and 24 species). The specificity of the Fla4/Fla2 primers was reported to be 100% (Hozbor et al., 1999) although, because the PCR was intended for use with human specimens, the bacterial and fungal species evaluated occur primarily in humans. Our findings of 100% specificity using bacterial genera and species commonly found in the swine respiratory tract provide an added measure of confidence regarding the use of these primers in investigation of swine PAR and bronchopneumonia.

The specificity of the *tox*A-7/*tox*A-6 primer set depends upon the premise that nontoxigenic strains lack the *tox*A gene. Results of the present study support this assumption, as well as data from many other investigations in which *tox*A-specific probes or alternative PCR targets within the gene were evaluated for identification of toxigenic isolates (Kamps et al., 1990a, 1990b; Frandsen et al., 1991; Foged, 1992; Donnio et al., 1999; Hunt et al., 2000). However, a few reports present conflicting information suggesting that silent or incomplete copies of *tox*A may occasionally be found in nontoxigenic strains (Kamps et al., 1990a; Hoskins and Lax, 1996; Kamp et al., 1996). When warranted, a phenotypic assay for PMT production can be performed to confirm a PCR positive result for the *tox*A-7/*tox*A-6 amplicon, e.g., if a toxigenic isolate is identified from an animal without clinical signs of atrophic rhinitis. Since the occurrence of nontoxigenic strains possessing *tox*A sequence appears to be a rare event, the *tox*A-7/*tox*A-6 primer set will very likely continue to provide a high level of

specificity even as the population of tested isolates increases.

The limit of detection for the multiplex PCR compares favorably to the limit of detection for individual primer sets, both as determined here and as reported previously by others. Alternative primer sets specific for the *tox*A gene were shown to require approximately 30–150 organisms (Kamp et al., 1996; Lichtensteiger et al., 1996) or 10 pg of DNA (Nagai et al., 1994) for a positive result, while the Fla4/Fla2 primer set was reported to require roughly 100 bacteria (Hozbor et al., 1999). The limit of detection for the KMT1T7/KMT1SP6 primer set was not previously examined (Townsend et al., 1998). Although clinical specimens were unavailable for inclusion in the present study, properly collected swabs obtained from infected swine would be expected to contain levels of bacteria in excess of the number required for visualization of amplicons using the multiplex PCR. However, diagnostic samples are frequently contaminated with mucous and blood which could interfere with recovery of bacteria and/or the PCR itself. Although not as convenient or rapid as direct testing of fluid recovered from swabs, a moderately high-throughput processing method in which DNA is extracted from swine nasal and tonsil swabs appears to overcome this problem (Kamp et al., 1996). Additional studies comparing the limit of detection for the multiplex PCR using swab samples processed by various methods would permit recommendations as to optimal protocols. At present, the PCR is recommended for evaluating material from cultures. A lysate can be prepared from a small area of heavy growth for use as template. Alternatively, we have found it convenient to visually screen plates for suspect colonies with characteristic morphology, which are then resuspended together in a single tube prior to boiling. The latter method may be preferable, particularly on plates with contaminating normal flora, since the small area of heavy growth sampled may not contain less predominant bacterial types.

Assay verification and validation are essential when implementing a new diagnostic procedure. Analytical verification is defined as the process of establishing performance characteristics including limit of detection, sensitivity, specificity, and reproducibility (Hoorfar and Cook, 2003; Nolte and Caliendo, 2003). The data reported here serve as analytical verification of the

multiplex PCR for identification of *B. bronchiseptica* and both toxigenic and nontoxigenic *P. multocida* from swine. This method is faster, more economical, and more objective than biochemical testing, particularly with respect to differentiating toxigenic from nontoxigenic *P. multocida*. While the multiplex PCR can be expected to provide clinically significant results with a high degree of sensitivity, specificity, and reproducibility, formal clinical validation using appropriate samples is required prior to routine implementation in a diagnostic laboratory.

Acknowledgements

The expert technical assistance of Michael Mullins and Pamela Recker is gratefully acknowledged. We are indebted to David Alt and the National Animal Disease Center Genomics Unit for DNA sequence data. Funding for K. deJong was provided by the Merck-Merial Veterinary Research Scholar Program, College of Veterinary Medicine, Iowa State University.

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